HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Exp Eye Res. 2016 January ; 142: 110–118. doi:10.1016/j.exer.2014.09.012.

The corneal fibrosis response to epithelial-stromal injury

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Abstract

The corneal wound healing response, including the development of stromal opacity in some eyes, is a process that often leads to scarring that occurs after injury, surgery or infection to the cornea. Immediately after epithelial and stromal injury, a complex sequence of processes contributes to wound repair and regeneration of normal corneal structure and function. In some corneas, however, often depending on the type and extent of injury, the response may also lead to the development of mature vimentin+ α-smooth muscle actin+ desmin+ myofibroblasts. Myofibroblasts are specialized fibroblastic cells generated in the cornea from keratocyte-derived or bone marrow-derived precursor cells. The disorganized extracellular matrix components secreted by myofibroblasts, in addition to decreased expression of corneal crystallins in these cells, are central biological processes that result in corneal stromal fibrosis associated with opacity or “haze”. Several factors are associated with myofibroblast generation and haze development after PRK surgery in rabbits, a reproducible model of scarring, including the amount of tissue ablated, which may relate to the extent of keratocyte apoptosis in the early response to injury, irregularity of stromal surface after surgery, and changes on corneal stromal proteoglycans, but normal regeneration of the epithelial basement membrane (EBM) appears to be a critical factor determining whether a cornea heals with relative transparency or vision-limiting stromal opacity. Structural and functional abnormalities of the regenerated EBM facilitate prolonged entry of epithelium-derived growth factors such as transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF) into the stroma that both drive development of mature myofibroblasts from precursor cells and lead to persistence of the cells in the anterior stroma. A major discovery that has contributed to our understanding of haze development is that keratocytes and corneal fibroblasts, but not myofibroblasts, produce large amounts of critical EBM components, such as nidogen-1, nidogen-2 and perlecan, that are essential for complete

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Proprietary interest statement: None of the authors have any proprietary or financial interests in the topics discussed in this manuscript.

regeneration of a normal EBM once laminin secreted by epithelial cells self-polymerizes into a nascent EBM. Mature myofibroblasts that become established in the anterior stroma are a barrier to keratocyte contributions to the nascent EBM. These myofibroblasts, and the opacity they produce, often persist for months or years after the injury. Transparency is subsequently restored when the EBM is completely regenerated, myofibroblasts are deprived of TGFβ and undergo apoptosis, and the keratocytes re-occupy the anterior stroma and reabsorb disordered extracellular matrix. The aim of this review is to highlight factors involved in the generation of stromal haze and its subsequent removal.

Keywords

Cornea; epithelial basement membrane; wound healing; myofibroblasts; bone marrow-derived cells; fibrocytes; stroma; haze; transforming growth factor-beta; platelet-derived growth factor

1. Introduction

The corneal wound healing response to epithelial-stromal injury involves a cascade of events that usually contribute to a return of normal stromal structure and function (Netto et al., 2005; Wilson, 2012). This response is a critical determinant of the outcomes of corneal surgical procedures, especially photorefractive keratectomy (PRK) and other corneal surface ablation procedures, in addition to scarring that may occur after injury, infection and other surgeries. In some cases, an abnormal wound healing response leads to loss of corneal transparency that is referred to as haze. One of the central concerns in corneal biology is the factors that promote normal corneal tissue regeneration rather than fibrosis (Netto et al., 2005).

Myofibroblast generation has been identified as the critical factor that leads to corneal stromal opacity or fibrosis (Jester et al., 1999b; Mohan et al., 2003; Wilson, 2012). Keratocytes are relatively quiescent stromal cells that function to maintain collagen and other extracellular matrix components (Singh et al., 2012; West-Mays and Dwivedi, 2006). Epithelial-stromal injury to the cornea initiates a complex stromal response that can lead to the development of mature myofibroblasts that express vimentin, alpha smooth muscle actin (αSMA) and desmin. These cells, that are themselves opaque due to limited corneal crystallin production (Jester et al., 1999b), produce enormous quantities of disorganized extracellular matrix once they become established in the anterior stroma and produce haze (Fig. 1) (Jester et al., 1999b; Mohan et al., 2003; Netto et al., 2006a; Stramer and Fini, 2004).

Myofibroblasts are fibroblastic cells that can be generated from either keratocyte-derived or bone marrow-derived precursor cells (Barbosa et al., 2010a; Novo et al., 2009; Saika et al., 2010; Singh et al., 2014b). Epithelial–stromal interactions modulate the generation of corneal myofibroblasts and the development of stromal opacity (Fini and Stramer, 2005; Wilson et al., 1999). The epithelial basement membrane plays a critical role in modulating myofibroblast development and persistence (Stramer et al., 2003; Torricelli et al., 2013a,b). Structural and functional defects in the regenerated epithelial basement membrane allow persistent penetration of transforming growth factor beta (TGF-β) and platelet-derived

growth factor (PDGF) from the epithelium into the anterior stroma at sufficient levels required for ongoing receptor activation in precursor cells (Kaur et al., 2009a; Netto et al., 2006a; Singh et al., 2014a; Torricelli et al., 2013b). TGF-β promotes development of mature myofibroblast development (Jester et al., 2002) and suppresses interleukin-1 (IL-1)-mediated apoptosis of mature myofibroblasts and their precursors (Barbosa et al., 2010b; Kaur et al., 2009a).

The purpose of the this review is to highlight the events that contribute to corneal wound healing after epithelial-stromal injury and describe the factors involved in stromal haze generation and restoration of transparency in corneas with haze.

2. Epithelial injury-induced stromal cell apoptosis

The corneal wound healing process, for example after photorefractive keratectomy (Rajan et al., 2004; Netto et al., 2006a; Torricelli et al., 2014), is mediated by autocrine and paracrine interactions of cytokines, growth factors, chemokines, and their receptors produced by epithelial, stromal, bone marrow-derived, and neural cells that contribute to remodeling and reestablishing normal corneal structure and function (Jester et al., 2002; Stramer et al., 2003; Wilson et al., 1999,2001). Anterior keratocyte apoptosis is the earliest change noted in the corneal stroma after injury or surgery, like PRK, in which there is trauma to the corneal epithelium (Helena et al., 1998; Wilson et al., 1996). Most commonly, cell death is detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, and with this assay the labeling typically peaks at approximately four hours after epithelial injury in mice and rabbits (Wilson et al., 1996, 2000). However, it has been demonstrated using transmission electron microscopy that the ultrastructural changes of apoptosis such as chromatin condensation, cell shrinkage, and budding of apoptotic bodies containing intracellular organelles and other contents begins in anterior keratocytes immediately after epithelial injury (Wilson et al., 1996). Keratocyte apoptosis is a controlled form of cell death that normally occurs without significant release of lysosomal enzymes or other intracellular components that damage the surrounding tissue or cells (Gavrieli et al., 1992; Netto et al., 2005; Wilson, 1998). Prior studies have suggested that injury-induced keratocyte apoptosis is mediated by release of pro-apoptotic cytokines from the epithelium (Barbosa et al., 2010b; Wilson and Kim, 1998). Cytokines implicated in this response include Fas ligand, interleukin-1 and tumor necrosis factor α (Wilson, 1998). For example, apoptosis of keratocytes at the sites of injection was noted in experiments in which mouse IL-1α was microinjected into the central stroma of mouse corneas (Wilson and Kim, 1998). Subsequent studies demonstrated that IL-1 stimulates keratocytes to produce Fas ligand messenger RNA and protein to trigger an apoptotic autocrine response (Mohan et al., 1997). Thus, keratocytes constantly produce the receptor Fas, but do not normally produce Fas ligand *in vivo* or *in vitro* in the absence of IL-1 receptor activation. Thus, when stimulated by IL-1, these cells produce both Fas ligand and Fas, and this simultaneous expression triggers apoptosis via autocrine mechanisms (Mohan et al., 1997). It is likely that the overall milieu and concentration of released IL-1, and other interacting cytokines, determines whether IL-1 triggers apoptosis or, alternatively, other functions such as chemokine and metalloproteinase production and hepatocyte growth factor and keratinocyte growth factor production in a particular keratocyte cell. The wave of stromal apoptosis normally lasts for

several days to a week after the injury and also includes the death of inflammatory cells and other bone marrow-derived cells, such as myofibroblast precursor fibrocyte cells, that penetrate the stroma from the limbal blood vessels beginning almost immediately after epithelial injury (Wilson, et al., 2004).

After the initial wave of keratocyte apoptosis, some cells dying in the stroma may also undergo necrosis. Necrosis is characterized by loss of plasma membrane integrity and random DNA degradation and is often associated with greater tissue injury and inflammatory response (Maldonado et al., 1996; Netto et al., 2007). Necrotic cells may also label with TUNEL assay, and, therefore, the cellular characteristics of necrosis are best identified by transmission electron microscopy.

3. Development of myofibroblast-associated stromal opacity

3.1. Precursor cells: Keratocyte-derived and bone marrow-derived myofibroblasts

Proliferation and migration of residual activated keratocytes in the peripheral and posterior stroma begins by 12 to 24 hours after corneal epithelial injury. Immunohistochemical testing for the antibody anti-Ki67, a marker of cells that are undergoing mitosis, can be used to detect the proliferation of cells surrounding the area of keratocyte apoptosis and necrosis (Gao et al., 1997; Helena et al., 1998; Mohan et al., 2003). These proliferating keratocytes give rise to corneal fibroblasts that repopulate the depleted stroma and can give rise to immature and fully differentiated myofibroblasts (Andresen and Ehlers, 1998; Chaurasia et al., 2009; Fini, 1999; Gan et al., 2001; Mohan et al., 2003; Musselmann et al., 2003). Simultaneously, thousands of bone marrow-derived cells migrate into the stroma from the limbal blood vessels, and possibly the tear film (Barbosa et al., 2010a; Wilson et al., 2004). Chemokines released from the injured epithelium and produced by keratocytes in response to cytokine stimulation attract inflammatory cells—such as macrophages/monocytes, T cells, and polymorphonuclear cells (Hong et al., 2001).

In many organs, including skin, liver lung and cornea, myofibroblasts can be generated from bone marrow-derived cells (Barbosa et al., 2010a; Bhawan and Majno, 1989; Direkze et al., 2003; Hashimoto et al., 2004; Scholten et al., 2011; Singh et al., 2014b). Thus, in a green fluorescent protein (GFP) chimeric mouse model in which only bone marrow-derived cells were GFP+, as many as 70% of SMA+ myofibroblasts that developed were shown to develop from bone marrow-derived precursor cells, with the balance of corneal myofibroblasts thought to be developed from keratocyte-derived precursor cells (Barbosa et al., 2010a; Singh et al., 2014b).

Bone marrow-derived myofibroblasts were also investigated in *in vitro* studies (Singh et al., 2012). In these experiments, bone marrow-derived cells and corneal stromal fibroblasts were obtained from mice with GFP expressed in all cells and normal GFP- control mice. GFP+ cells of one type were co-cultured with GFP- cells of the other type in Primaria plates to monitor juxtacrine signaling or in Transwell plates to monitor paracrine signaling. That study demonstrated conclusively that both bone marrow-derived cells and corneal stromal fibroblasts could transform into myofibroblasts (Fig. 2). Interestingly, the percentage of αSMA+ myofibroblasts generated from either precursors cell type was significantly higher

when both cells were co-culture together (juxtacrine) as compared to when bone marrow-derived cells or mouse corneal stromal fibroblast were culture in different compartments of Transwell System (paracrine) (Singh et al., 2012). Thus, juxtacrine interaction between the two precursor cell types could be important in the generation of large numbers of functional myofibroblasts in the cornea *in situ*. Our working hypothesis is that corneal myofibroblasts are generated from both keratocytes-derived and bone marrow-derived precursor cells, and the type of myofibroblast that predominates in a particular injured cornea, may be related to the type and extent of injury, genetic factors, and other unknown influences (Stepp et al., 2014; Wilson, 2012).

3.2. Development of mature myofibroblasts

One of the intriguing observations about haze development in the cornea is the time course of appearance of the haze and associated myofibroblasts after haze-inducing injury. Severe “late haze” that is clinically significant and associated with regression of the surgical correction is typically detectable by slit lamp biomicroscopy at around one month after PRK in humans and reaches a peak at three or four months after surgery (Raviv et al., 2000). In rabbits the slit lamp appearance of haze follows a similar course after PRK surgery, although some myofibroblasts that give rise to haze can be detected by immunohistochemical staining as early as two weeks after surgery (Chaurasia et al., 2009; Netto et al., 2006a).

Myofibroblasts have been shown to have variable cell phenotypes based on immunohistochemical staining of intermediate filament (IF) proteins (Chaurasia et al., 2009; Kohnen et al., 1996; Schmitt-Graff et al., 1994). The IF proteins vimentin and desmin, along with actin microfilaments, microtubules, and their associated proteins constitute a cytoskeleton system in the human cornea. These components interact with each other to form a meshwork which is essential for normal growth, differentiation, integrity, and function of corneal cells (Kivela and Uusitalo, 1998). Vimentin is a major class of the IF proteins in mesenchymal cells such as fibroblasts, vascular endothelial cells, and adipocytes (Lazarides, 1980b). Desmin is expressed at high levels in striated muscle cells, myocardium, and most smooth muscle cells (Lazarides, 1980a). Although there is basal expression of vimentin in keratocytes in the cornea (Ishizaki et al., 1993; Kivela and Uusitalo, 1998; Mimura et al., 2008; Wollensak and Witschel, 1996), constitutive expression of desmin is not observed in keratocytes in unwounded corneas (Ishizaki et al., 1993; Kivela and Uusitalo, 1998). Based on the expression of such IF proteins in myofibroblasts a classification system has been proposed (Kohnen et al., 1996; Schmitt-Graff et al., 1994). Thus, myofibroblasts that express only vimentin are termed V-type myofibroblasts, those that express vimentin and desmin are called VD-type myofibroblasts, and those that express vimentin, SMA, and desmin are called VAD-type myofibroblasts.

Chaurasia et al (2009) analyzed the expression of these IF proteins in rabbit corneas destined to develop haze after PRK surgery. That study revealed that after −9.0 diopter PRK there is an increase in stromal cells expressing vimentin at higher levels in the anterior stroma (V-type myofibroblasts) by one week after surgery. Some of these cells subsequently begin to express SMA (VA-type myofibroblasts) at about two weeks after surgery, but don't yet express desmin. The number of VA-type myofibroblasts in the anterior stroma increases

with time and then at around two weeks after surgery some of these cells also begin to express desmin (VAD-type myofibroblasts). By 4 weeks after −9D PRK, when haze at the slit lamp is becoming prominent, almost all of the myofibroblasts in the anterior stroma are the most mature VAD-type myofibroblasts. Thus, corneal myofibroblast precursors, either keratocyte-derived or bone marrow-derived, undergo an ordered differentiation from V-type cells, to VA-type cells, and finally to mature VAD-type cells during the healing response to corneal injury associated with haze.

3.3. The role of the epithelial basement membrane and growth factors in haze formation

Many factors likely contribute to myofibroblast generation and haze formation after PRK surgery. Some of the factors include the length of time required for epithelial defect healing, the depth of the ablation, irregularity of postoperative stromal surface, and removal of the epithelial basement membrane (Kuo et al., 2004; Moller-Pedersen et al., 1998; Stramer et al., 2003; Torricelli et al., 2013a).

After surface ablation surgeries, like PRK, mature myofibroblasts may be generated in the anterior stroma immediately beneath the epithelial basement membrane (Mohan et al., 2003). These cells are meant to function as beneficial contributors to corneal wound healing, producing innumerous extracellular matrix components, including type I collagen and fibronectin (Gao et al., 2008; Karamichos et al., 2010). However, if mature myofibroblasts develop in great numbers, a large amount of disorganized extracellular matrix is secreted and contributes to stromal opacity in the cornea. In addition, the myofibroblasts themselves are opaque relative to keratocytes due to diminished crystallin protein production (Jester et al., 2012,1999b; Stramer and Fini, 2004). It is important to distinguish pathological late haze attributable to myofibroblasts, which is a persistent corneal opacity observed at one to three months after PRK and lasting from 6 months to years, from the mild, clinically insignificant haze that occurs in the first weeks in almost all corneas that undergo PRK. The latter haze is attributable to corneal fibroblasts generated from keratocytes that are also opaque due to decreased corneal crystallin production (Jester et al., 1999b,2008,2012) but these cells produce less disordered extracellular matrix materials. The latter haze is milder and transient —tending to disappear within a few months.

Late haze is more common after PRK surgery for higher corrections for myopia. Mohan et al (2003) have shown that keratocytes apoptosis, keratocytes proliferation, and myofibroblast cell density are more pronounced after high myopia PRK (−9.0D) correction than moderate myopia PRK (−4.5D) correction in the rabbit model (Fig. 3) In addition, Netto et al (2006a) demonstrated a relationship between the level of corneal haze formation after PRK and the level of stromal surface irregularity. In that study, −4.5 D PRK, an amount of laser ablation usually insufficient to generate persistent mature myofibroblasts and haze, was performed after removal of the epithelium. However, in different groups of rabbits, a fine mesh screen was placed in the path of the excimer laser beam for the last 10%, 30% and 50% of the pulses of the PRK ablation. This masking of the excimer laser beam produced graduated and reproducible stromal surface irregularities. Myofibroblast density and the severity of haze were directly related to the degree of induced surface irregularity (Netto et al., 2006a). In addition, the epithelial basement membrane had more

apparent defects detected with light microscopic immunohistochemistry as the level of surface irregularity increased. It was hypothesized that these basement membrane defects altered barrier function and allowed greater and persistent levels of growth factors such as TGFβ and PDGF derived from the epithelium to penetrate into the anterior stroma to drive development of myofibroblasts from precursor cells (Netto et al., 2006a; Wilson, 2012). Thus, this hypothesis holds that the epithelial basement membrane is an integral corneal regulatory structure that limits the fibrotic response by regulating the availability of epithelial derived TGF-β and PDGF, and perhaps other cytokines and extracellular components, to stromal cells, including myofibroblast precursors (Torricelli et al., 2013b; Wilson, 2012).

TGF-β has long been appreciated as a critical modulator of corneal myofibroblast development *in vitro* (Fini, 1999; Funderburgh et al., 2001; Jester et al., 1999a; Masur et al., 1996; Petridou et al., 2000) and *in vivo* (Jester et al., 1997). The *in vivo* sources for high levels of TGF-β that can stimulate stromal myofibroblast development is the corneal epithelium, and in the early postoperative period, before the epithelial defect closes, likely the tears (Vesaluoma et al., 1997; Wilson et al., 1994). TGF-β plays an essential role in wound healing through its pleiotropic effects on cell proliferation and differentiation, extracellular matrix production, and immune modulation (Finnson et al., 2013; Penn et al., 2012). It is recognized that TGF-β is released in a latent complex formed by three proteins: TGF-β, the processed TGF-β pro-peptide, and a member of the latent TGF-β binding protein (LTBP) family. LTBPs are microfibril-associated proteins that tether latent TGF-β to extracellular matrix (Munger and Sheppard, 2011). Moreover, TGF-β activation seems to be an important checkpoint controlling TGF-β's action. Latent TGF-β activators include proteases (Ge and Greenspan, 2006; Yu et al., 2013), thrombospondim-1 (Schultz-Cherry and Murphy-Ullrich, 1993), reactive oxygen species (ROS) (Barcellos-Hoff and Dix, 1996) and integrins (Horiguchi et al., 2012; Munger et al., 1999; Wipff and Hinz, 2008). There are also three TGF-β isoforms (TGF-β 1-3) encoded by separate genes (Munger and Sheppard, 2011). TGF-β isoforms play distinct functions in wound healing with TGF-β1/2 having predominantly pro-scarring roles and TGF-β3 having mainly anti-scarring effects (Finnson et al., 2013; Karamichos et al., 2014; Matsuba et al., 2011).

PDGF is another cytokine that has been shown to be an important modulator of myofibroblast development in the cornea (Jester et al., 2002; Kaur et al., 2009b; Singh et al., 2011,2014a; Stramer and Fini, 2004). IL-1 produced by stromal cells has been shown to trigger apoptosis of myofibroblasts when TGF-β is withdrawn from the cells (Barbosa et al., 2010b; Kaur et al., 2009a).

Recent studies (Torricelli et al., 2013a) assessed the ultrastructure of the epithelial basement membrane (EBM) using transmission electron microscopy in rabbit corneas with and without haze. Two groups of rabbits underwent PRK surgery, the first group had a −4.5D PRK ablation that usually doesn't generate haze and the second group had −9.0D PRK ablation that uniformly generates severe haze. At one month after surgery, immunohistochemical assay was performed to detect αSMA+ cells and transmission electron microscopy was performed to evaluate the EBM. Interestingly, an absence of normal EBM regeneration was noted in all rabbit corneas at one month after −9.0D PRK

(corneas with haze) without regeneration of the lamina lucida and lamina densa layers characteristic of mature EBM. Conversely, in rabbit corneas that had moderate −4.5D PRK, the EBM was fully regenerated with morphology similar to that in unwounded control corneas (Fig. 4), except in one animal. In that cornea, small areas of haze and myofibroblasts with disordered EBM were intermingled with transparent areas without myofibroblasts that had normal EBM. Thus, in areas of corneas with normal regeneration of the EBM there were no myofibroblasts and the stroma was transparent.

Recent work in our laboratory has demonstrated conclusively that keratocytes and corneal fibroblasts produce some of the key EBM components such as nidogen-1, nidogen-2 and perlecan (A. Santhanam, A.A.M Torricelli, J. Wu, S.E. Wilson, unpublished data, 2014). While epithelial cells can also produce these components, our work suggests that after the self-polymerizing laminin layer is laid down early after injury, deeper EBM components, including those associated with the lamina lucida and lamina densa, must be provided by keratocytes and/or corneal fibroblasts. Our findings extend observations in previous studies (Kenney et al., 1997; Maguen et al., 2007, 2008) about production of basement membrane components by stromal cells in the cornea. Maguen et al (2008) reported an accumulation of nidogen-2 and perlecan around intrastromal corneal rings inserted in the stroma for post-laser in situ keratomileusis keractasia and keratoconus. Other studies also found an abnormal deposition of stromal extracellular matrix and basement membrane components (type IV collagen, perlecan, laminin-1, nidogen) in areas of corneas with subepithelial fibrosis (Kenney et al., 1997; Ljubimov et al., 1996). Our working hypothesis is that the prolonged recovery of the EBM is attributable to a deficiency of EBM components and/or signaling from keratocytes or corneal fibroblasts that occurs in higher PRK corrections. Early after PRK, this deficiency is attributable to the greater wave of keratocyte apoptosis noted in high PRK corrections (Mohan et al., 2003) and, possibly in humans, genetic differences between individuals who get late haze and those that don't after high PRK correction. The resulting defective or delayed regeneration of EBM allows prolonged penetration of high levels of TGF-β and PDGF into the stroma from the regenerating epithelium that drive myofibroblast generation from precursor cells (Singh et al., 2012,2014a). It appears myofibroblasts do not contribute the necessary components to complete the assembly of the basement membrane; and their ongoing development, along with the large amount of disorganized extracellular matrix they produce, further hinders keratocytes from reoccupying a position in the anterior stroma where they can contribute to the full recovery of the EBM (Fig. 4). As a result, a vicious cycle is established in corneas with persistent haze in which myofibroblasts are maintained by the epithelium in the absence of a functional basement membrane; and myofibroblasts themselves along with the disorganized matrix they produce, provide a barrier against keratocytes that are present deeper in the stroma moving into a subepithelial position to contribute to basement membrane regeneration. Only when this cycle is in some way interrupted, which may occur gradually over months to years, is the basement membrane fully regenerated. At this point, EBM barrier function is reestablished, stromal levels of TGF-β and PDGF fall, myofibroblasts undergo apoptosis, keratocytes reoccupy the anterior stroma, abnormal extracellular matrix is reabsorbed and corneal transparency is restored (Torricelli et al., 2013a). Often, as late haze resolves, clear areas called “lacunae” appear at the edges or in the center of haze (Fig. 5). These lacunae likely represent areas

where the EBM has been repaired, myofibroblasts have undergone apoptosis and keratocytes have reoccupied the anterior stroma and reabsorbed the disordered extracellular matrix produced by the myofibroblasts. Over time, typically many months to years, these lacunae enlarge and coalesce and frequently corneal transparency is completely restored.

Importantly, it is likely that these events are initiated in most corneas that have epithelial-stromal injuries. Thus, TGFβ and PDGF begin penetrating into the anterior stroma from injured epithelium and tears immediately after the insult, bone marrow-derived invade the stroma, and both bone marrow-derived and keratocyte-derived precursors begin to undergo development into myofibroblasts. However, in most corneas with epithelial abrasions and lower-correction PRKs, or even the majority of animal and human corneas that have high-correction PRK, perhaps at least partially because the initial wave of apoptosis is not as extensive, corneal fibroblasts and keratocytes repopulate the anterior stroma in sufficient numbers to contribute essential EBM components to regenerate a normal fully-functional EBM within a week or two after surgery. Regeneration of the EBM leads to a drop in TGFβ and PDGF levels in the anterior stroma, immature developing myofibroblasts undergo apoptosis, and the anterior stroma is completely repopulated with keratocytes. Thus, in every cornea that has epithelial injury, a race of sorts is initiated between keratocytes/corneal fibroblasts and myofibroblasts to repopulate the anterior stroma. If the keratocytes/corneal fibroblasts win that race, a normal EBM is regenerated and transparency is maintained. If the myofibroblasts win that race, an abnormal EBM persists and haze develops in the cornea. In some corneas, the keratocytes win the race in one area and myofibroblasts win in another area. In the latter cases, areas of opacity are interspersed with areas of transparency.

3.4. Small leucine-rich proteoglycans in corneal wound healing

The family of small leucine-rich proteoglycans (SLRPs) contains several extracellular matrix molecules that are structurally related by a protein core composed of leucine-rich repeats covalently linked to glycosaminoglycan (GAG) side chains (McEwan et al., 2006). SLRPs have been shown to play a role in corneal wound healing and homeostasis (Chakravarti, 2002; Funderburgh et al., 1998; Mohan et al., 2011a). Decorin, biglycin, fibromodulin, mimecan, keratocan, and lumican are some of the SLRPs expressed in the cornea (Mohan et al., 2011a).

SLRPs are implicated in regulating assembly of collagen fibrils and the highly organized extracellular matrix essential for corneal transparency (Chakravarti et al., 2006). Lumicans are thought to regulate collagenous matrix assembly in connective tissues via their bi-functional character. Thus, the protein moiety of lumican binds collagen fibrils at strategic loci and the highly charged hydrophilic glycosaminoglycans regulate interfibrillar spacing (Funderburgh et al., 1995; Hassell et al., 1983; Kao and Liu, 2002). Chakravarti et al (1998) have demonstrated that corneal opacity develops in Lum−/− mice and electron microscopic examination revealed abnormally thick collagen fibers, especially in the posterior stroma. This haze is associated with the presence of a disorganized collagenous matrix with larger fibril diameters and disorganized fibril spacing (Chakravarti et al., 1998,2002; Kao and Liu, 2002).

Decorin is another SLRP that also plays an important role in maintaining the physical characteristics essential for corneal transparency including interfibrillar spacing regularity and a very small collagen fibril diameter (Michelacci, 2003; Mohan et al., 2011b). In addition, Mohan et al (2011a) have found that decorin can inhibit scar formation in the cornea, presumably by hindering TFGβ activity. Decorin works as endogenous inhibitor of TFGβ and it has been shown to regulate TFGβ signal transduction pathway through its interaction with low-density lipoproteins receptor-related protein-1 and decorin endocytic receptor (Kalamajski and Oldberg, 2010; Mohan et al., 2011a, b). However, decorin also modulates signaling of epidermal growth factor and insulin-like growth factor and its receptor, so the precise mechanism through which decorin can reduce corneal opacity after a treatment such as PRK remains to be determined.

4. Mitomycin C and breakthrough haze in corneal wound healing

The type and level of stromal opacity that develops after PRK is not clinically significant in the eyes of most patients. However, in some eyes, especially after high levels of correction for myopia, severe opacity develops. The introduction of mitomycin C (MMC) has had a major impact on PRK and other surface ablation surgeries. MMC is classified as an alkylating agent and acts in all phases of the cell cycle. It crosslinks DNA between adenine and guanine thereby inhibiting DNA synthesis (Wilson et al., 1997). MMC, therefore, works as a genotoxic drug, and at high concentrations, cellular RNA and protein synthesis are also suppressed (Fujita et al., 1997; Galm et al., 2005; Goeptar et al., 1994; Santhiago et al., 2012; Verweij and Pinedo, 1990; Yu et al., 2013).

The most important corneal effects of MMC after surface ablation surgery are inhibition of the proliferation of keratocytes and other cells in the stroma, and the resulting inhibition of proliferation of large numbers of myofibroblast precursor cells (Netto et al., 2006b), although a limited increase in anterior keratocyte apoptosis is also noted (Chang, 2005; Kim et al., 2003; Netto et al., 2006b). Thus, Netto et al (2006b) evaluated the effect of MMC on corneal apoptosis, cellular proliferation, haze, and keratocyte density in rabbit corneas. These authors showed the predominant effect of MMC was at the level of blocked replication of keratocytes and other progenitor cells of myofibroblasts. This study also found a persistent decrease in keratocyte density in the anterior stroma after MMC treatment.

5. Alternative treatments for PRK-induced corneal haze

Although MMC has been highly effective in preventing late haze development when applied during PRK surgery, the long-term effects still remain as an issue of concern (Wilson, 2012). A decrease in keratocyte density and the relatively short follow up on corneas treated with this powerful medication raise concerns about potential long-term effects of the treatment (de Benito-Llopis et al., 2012; Netto et al., 2006b). It is important to note that apparent normalization of keratocyte density in the stroma of corneas assessed with histologic methods or confocal microscopy does not guarantee late functional normality of the repopulating stromal cells (Wilson, 2012). Could a patient treated with mitomycin C develop stromal abnormalities such as necrosis 30 or 50 years later? Mitomycin C has been

used in PRK for only about 15 years and, therefore, we must wait to conclusively answer this question.

A drug that could specifically block myofibroblast development from precursor cells without affecting keratocytes and other stromal cells would be an optimal approach to inhibit haze formation (Wilson, 2012). Several studies tested pharmacological agents to replace MMC treatment after PRK surgery. Moller-Perdersen et al (1998) found that neutralizing antibodies to TGF-β could inhibit stromal haze after PRK but this approach was never developed for clinical use. Santhiago et al (2011) observed in a rabbit model that PRM-151, a monocyte development inhibitor, could decrease corneal myofibroblast generation when applied by subconjunctival injection for several days. Unfortunately, topical use of PRM-151 was not effective, likely due to limited penetration of the intact epithelium. Torricelli et al (2014, in press) investigated whether resolvin E1 (RvE1), a lipid-derived immuno-modulator, could regulate the development of corneal haze and opacity-related myofibroblasts after opacity-generating high correction PRK in rabbits. This masked study showed that topical 0.1% RX-10045 given immediately after laser application and every four hours for 5 days after opacity-inducing −9D PRK decreased haze generation in the rabbit cornea. However, the drugs effect on myofibroblast generation was only partial since RX-10045 appeared to only block myofibroblast generation from bone marrow-derived precursors without substantial effect on myofibroblast generation from keratoyte-derived precursors. However, RX-10045 could be effective if combined with another compound that blocked the keratocyte-derived pathway. Mohan et al (2010) showed that decorin gene transfer inhibited TGFβ-mediated myofibroblast development from corneal fibroblasts in vitro and suggested that decorin gene therapy could be used in the treatment of corneal haze in vivo (Mohan et al., 2011)—although the difference in SMA+ myofibroblasts between corneas that had decorin gene transfer compared to controls was unlikely to be clinically significant based on immunohistochemistry provided in this study. Studies continue to try to identify a drug or combination of drugs that can be used to specifically inhibit myofibroblast generation in the cornea.

Acknowledgments

Supported in part by US Public Health Service grants EY10056 and EY015638 from the National Eye Institute, National Institutes of Health, Bethesda, MD and Research to Prevent Blindness, New York, NY.

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Highlights Steven E. Wilson

- The level of keratocyte apoptosis is important in corneal opacity
- Keratocytes produce key corneal epithelial basement membrane components
- Competition between myofibroblasts and keratocytes is critical in haze development

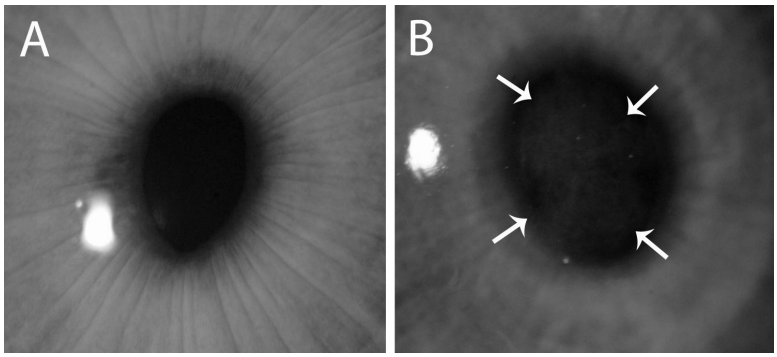

Figure 1.

Slit lamp corneal photographs. **A.** Clear unwounded rabbit cornea **B.** Subepithelial corneal haze in a rabbit model at one month after high myopia PRK correction. Magnification 16X.

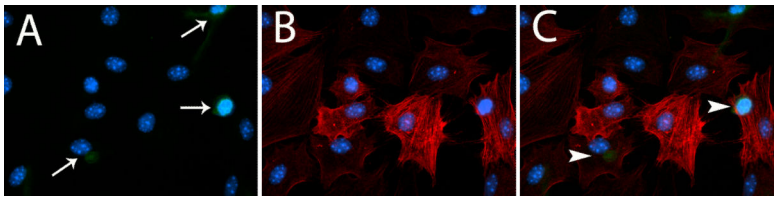

Figure 2.

Representative immunocytochemistry for alpha-smooth muscle actin (αSMA) in GFP+ bone marrow-derived cells when cultured in the same well with normal GFP− mouse corneal stromal fibroblasts after 4 day of transforming growth factor β treatment. The nuclei of all cells were stained blue with DAPI in each panel. **A.** green-stained GFP+ cells (arrows). **B.** red αSMA+ cells staining. **C.** overlay of the DAPI, alpha smooth muscle actin and GFP staining. Note that some GFP+ cells are also αSMA+, indicating bone marrow-derived cells that differentiated into myofibroblasts (arrowheads). Magnification 40X.

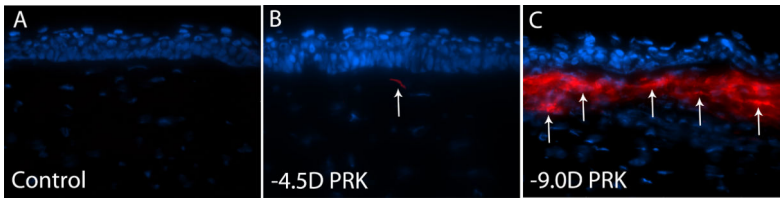

Figure 3.

Representative images of immunohistochemical staining for the alpha-smooth muscle actin (αSMA) marker for myofibroblasts in the central cornea of rabbit corneas at one month after PRK. **A.** A control cornea that did not have PRK. **B.** A cornea at one month after −4.5D PRK surgery. **C.** A cornea at one month after −9.0D PRK surgery. Note the limited number of αSMA+ (arrow) present after −4.5D PRK compared to a high density of αSMA+ (arrows) in the subepithelial stroma of the cornea that had-9.0D PRK and developed severe haze. Cell nuclei are stained blue with DAPI and SMA+ cells are stained red (arrows). Magnification 400x.

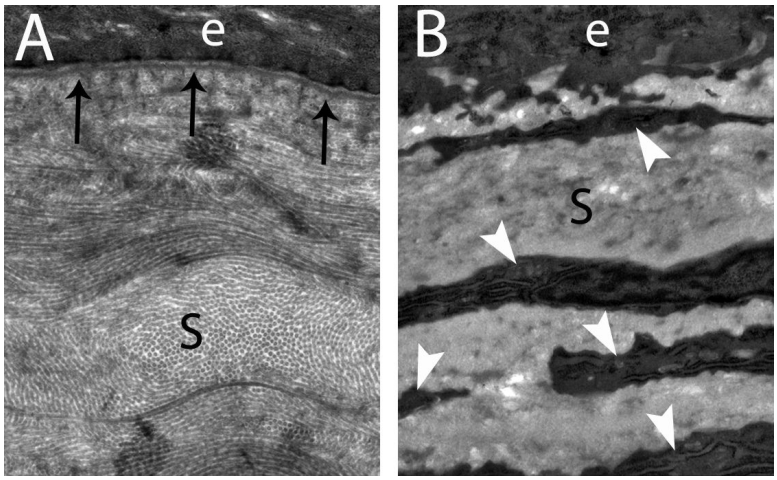

Figure 4.

Representative transmission electron microscopy (TEM) images of rabbit corneas (e, epithelium; S, stroma). **A.** A clear cornea at 2 weeks after −4.5D PRK had normal regeneration of the epithelial basement membrane (arrows) with clear lamina densa and lamina lucida. **B.** A cornea with haze at even 4 weeks after −9.0D PRK showed a complete absence of regenerated epithelial basement membrane beneath the epithelium, although it is likely there is a nascent EBM consisting of laminin and some other components of EBM beneath the basal epithelial cells. Also note the large numbers of myofibroblasts (cells indicated with arrowheads with prominent endoplasmic reticulum) in the anterior stroma surrounded by a disorganized extracellular matrix that would act as a barrier to keratocytes repopulating the anterior stroma. 13,000X magnification

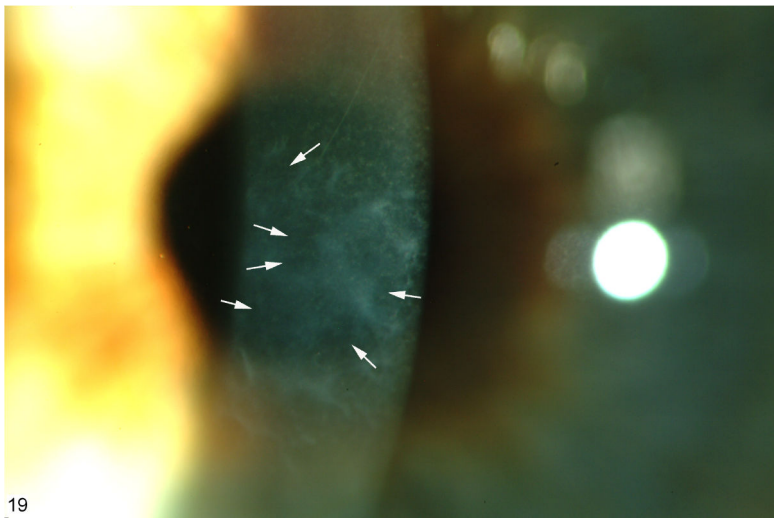

Figure 5.

Human cornea with severe late haze at over one year after −8 diopter PRK in which mitomycin C was not used after surgery. Arrows indicate clear “lacunae” appearing in the haze that continued to enlarge over time and eventually coalesce to yield a completely clear cornea by three years after PRK. 20X magnification.